Foxo-mediated *Bim* transcription is dispensable for the apoptosis of hematopoietic cells that is mediated by this BH3-only protein

Marco J Herold[1,2] , *Leona Rohrbeck[1,2], *Mathias J Lang[1,2,3], Raelene Grumont[4], Steve Gerondakis[4], Lin Tai[1,2], Philippe Bouillet[1,2], #Thomas Kaufmann[1,5] and #^Andreas Strasser[1,2]

[1] The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia
[2] Department of Medical Biology, The University of Melbourne, Melbourne, Victoria, Australia
[3] current address: Biocentre, University of Basel, Basel, Switzerland
[4] Australian Centre for Blood Diseases and Department of Clinical Hematology, Central Clinical School, Monash University, Melbourne, Victoria, Australia.
[5] current address: Institute of Pharmacology, University of Bern, Bern, Switzerland
* LR and MJL contributed equally to this work
# TK and AS share senior authorship
^ address for correspondence: Andreas Strasser, The Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville VIC 3052, Australia, ph: +61-3-9345-2624, FAX: +61-3-9347-0852, email: strasser@wehi.edu.au

Running title: Foxos are not critical for the activation of Bim

Keywords: Bim; apoptosis; Bcl-2; BH3-only proteins; hematopoiesis

Abstract

The BH3-only protein Bim is a critical initiator of apoptosis in hematopoietic cells. Bim is up-regulated in response to growth factor withdrawal and over-expression studies have implicated the transcription factor Foxo3a as a critical inducer. To test this hypothesis *in vivo*, we generated mice with mutated Foxo binding sites within the *Bim* promoters ($Bim^{\Delta Foxo/\Delta Foxo}$). Contrary to Bim-deficient mice, $Bim^{\Delta Foxo/\Delta Foxo}$ mice had a normal hematopoietic system and, upon cytokine withdrawal, diverse hematopoietic cells from these mice died at a normal rates. These results indicate that regulation of *Bim* by Foxo transcription factors is not critical for the killing of hematopoietic cells.

Introduction

The "Bcl-2-regulated" apoptotic pathway plays a critical role in the hematopoietic system[1]. It is controlled by the complex interactions of three sub-groups of the Bcl-2 protein family: the pro-survival Bcl-2-like proteins, the Bax/Bak multi-BH domain pro-apoptotic proteins and the pro-apoptotic BH3-only proteins[2]. Stress stimuli, such as growth factor deprivation, cause induction of BH3-only proteins[3]. These proteins then activate Bax/Bak, either through direct binding and/or indirectly by binding to the pro-survival Bcl-2-like proteins[4]. Activated Bax/Bak trigger mitochondrial outer membrane permeabilisation (MOMP), which unleashes the caspase cascade that causes cell demolition[2].

The BH3-only proteins are essential for the initiation of apoptosis and different members of this subgroup are critical for cell death triggered by distinct apoptotic stimuli[2]. Bim[5] is a critical regulator of hematopoietic cells[6, 7]: it is, for example, essential for

the deletion of auto-reactive T[8] and B cells[9] and the killing of effector T and B lymphocytes during shut-down of immune responses[10, 11].

Experiments using dominant negative or constitutively active mutants of Foxo3a implicated this transcription factor as a critical inducer of Bim in apoptosis triggered by cytokine deprivation[12-14]. To explore the physiological relevance of this process, we generated mice in which all Foxo binding sites within the *Bim* promoters[15] had been mutated (called $Bim^{\Delta Foxo/\Delta Foxo}$ mice). Contrary to Bim-deficient mice, $Bim^{\Delta Foxo/\Delta Foxo}$ and $Foxo3a^{-/-}$ mice had a normal hematopoietic system. Moreover, in contrast to Bim-deficient cells diverse lymphoid cell subsets and mast cells from $Bim^{\Delta Foxo/\Delta Foxo}$ mice were normally sensitive to cytokine deprivation. These results indicate that direct transcriptional induction of *Bim* by Foxo is not critical for homeostasis and cytokine deprivation-induced apoptosis of hematopoietic cells.

Results

Identification and validation of Foxo binding sites in the Bim promoters

Previous over-expression studies showed that Foxo3a can enhance Bim expression by binding to two specific sequence motives (GTAAACAA) within its two promoters[12-14]. Since Bim is critical for cytokine deprivation-induced apoptosis in diverse hematopoietic cell subsets[6, 16], it was concluded that growth factor withdrawal kills cells through transcriptional upregulation of Bim by Foxo3a[12-14].

Our bioinformatics analysis of the *Bim* gene revealed the two published[12-14] and two additional Foxo3a binding sites (-625 and +2509 from Exon1) (FIG 1A). To examine whether Foxo3a can bind to these sites, we performed EMSA with nuclear extracts from

293T cells that transiently expressed a tamoxifen (4-OHT) inducible HA tagged Foxo3a (HA-Foxo3a(A3)ER) (Figure 1B). We only observed reproducible Foxo3a-specific binding to site 4; this band disappeared when nuclear extracts containing HA-Foxo3A(A3)ER were incubated with HA- or Foxo3A-specific antibodies (FIG 1B & C). Moreover, we could efficiently compete Foxo3A binding to site 4 with unlabeled, but not with mutated oligos (supplementary FIG 1). These results reveal that site 4 in the mouse *Bim* promoters is the dominant site bound by Foxo3a.

$Bim^{\Delta Foxo/\Delta Foxo}$ mice have a normal hematopoietic system

Foxo3a belongs to a transcription factor family with overlapping expression patterns and binding to target sequences in DNAexpression[17]. To examine the physiological role of Foxo driven Bim activation, we generated mice in which the invariable core sequence of the common Foxo binding sequences in the *Bim* promoters were exchanged from AACA to TCGA or GTGG for site 1 or sites 2, 3 and 4, respectively (supplementary FIG 2A & B). To verify the functional impact of these mutations, we generated T cell blasts from wt and $Bim^{\Delta Foxo/\Delta Foxo}$ mice and ectopically expressed HA-Foxo3a(A3)ER; this elicited *Bim* mRNA upregulation only in wt but not in $Bim^{\Delta Foxo/\Delta Foxo}$ T cells treated for 24 h with 4-OHT (supplementary FIG 2C). Finally, we observed no compensatory Foxo3a or Foxo1 protein upregulation in thymocytes from $Bim^{\Delta Foxo/\Delta Foxo}$ mice (supplementary FIG 2D).

We examined the hematopoietic system of the $Bim^{\Delta Foxo/\Delta Foxo}$ mice using wt, $Foxo3a^{-/-}$ and Bim deficient animals as controls. As previously reported[6], $Bim^{-/-}$ mice had a marked increase of mature $CD4^+8^-$ and $CD4^-8^+$ thymocytes compared to wt controls (FIG 2A). In contrast, $Bim^{\Delta Foxo/\Delta Foxo}$ and $Foxo3a^{-/-}$ mice had normal thymic cell subset distribution

(FIG 2A). *Bim* mRNA and Bim protein levels in thymocytes from $Bim^{\Delta Foxo/\Delta Foxo}$ and $Foxo3a^{-/-}$ mice were comparable to those seen in wt thymocytes (FIG 2B, C).

Similarly, only $Bim^{-/-}$ mice but not the $Bim^{\Delta Foxo/\Delta Foxo}$ or $Foxo3a^{-/-}$ animals had abnormally increased numbers of T as well as B cells and granulocytes in the spleen (FIG 3A). Accordingly, spleen cells from $Bim^{\Delta Foxo/\Delta Foxo}$ and $Foxo3a^{-/-}$ mice expressed normal levels of *Bim* mRNA and Bim protein (Figures 3B, C). Based on these results, we conclude that Foxo-mediated transcriptional activation of Bim is not essential for the normal developmentally programmed death of hematopoietic cells.

Foxo-mediated transcriptional activation of Bim is dispensable for killing of thymocytes

Bim is critical for thymocyte apoptosis triggered by diverse cytotoxic insults[6]. To examine the contribution of Foxo-mediated transcriptional induction of *Bim* in this scenario, we isolated thymocytes from $Bim^{\Delta Foxo/\Delta Foxo}$, $Foxo3a^{-/-}$, $Bim^{-/-}$ and wt animals, and cultured them in the absence of cytokines or treated them with ionomycin and dexamethasone. Phorbol ester (PMA) an apoptotic stimulus that does not depend on Bim[6] served as a control. The $Bim^{-/-}$ and to a lesser extent the $Foxo3a^{-/-}$ thymocytes showed abnormally increased survival in simple medium, but the $Bim^{\Delta Foxo/\Delta Foxo}$ thymocytes died at the same rate as their wt counterparts (FIG 4). As previously reported[6], loss of Bim substantially reduced ionomycin induced killing of thymocytes, but cells from the $Bim^{\Delta Foxo/\Delta Foxo}$ and $Foxo3a^{-/-}$ mice behaved like wt thymocytes (FIG 4). Finally, thymocytes from $Bim^{-/-}$ and to a lesser extent those from $Foxo3a^{-/-}$ mice survived treatment with dexamethasone better than cells from the wt or $Bim^{\Delta Foxo/\Delta Foxo}$ mice (FIG 4).

Foxo-mediated transcriptional activation of Bim is dispensable for cytokine withdrawal induced apoptosis of hematopoietic cells

Previous studies showed that cytokine deprivation activates Foxo3a and that the resulting apoptosis can be inhibited by expression of a dominant negative Foxo3a[12-14]. It was therefore concluded that Foxo3a-mediated transcriptional induction of *Bim* is essential for cytokine deprivation induced apoptosis.

Mature T cells require IL-7 for survival[18] and the cytokine deprivation-induced death of these cells requires Bim[6]. To examine the role of Foxo-mediated transcriptional upregulation of *Bim* in this setting, we isolated $CD4^+$ and $CD8^+$ T cells from the spleens of $Bim^{\Delta Foxo/\Delta Foxo}$, $Foxo3a^{-/-}$, $Bim^{-/-}$ and wt mice and cultured them in the presence or absence of IL-7. $CD4^+$ as well as $CD8^+$ T cells of all genotypes survived well in the presence of IL-7, but only the $Bim^{-/-}$ T cells showed extended survival in the absence of this cytokine, whereas the $Foxo3a^{-/-}$ and $Bim^{\Delta Foxo/\Delta Foxo}$ T cells died at similar rates as the wt cells (FIG 5A, upper panel).

Bim is also critical for cytokine deprivation-induced apoptosis in mature B cells[6]. When FACS-sorted transitional (IgM^+IgD^+) and follicular ($IgM^{low}IgD^+$) B cells from the spleens were cultured in simple medium, only the $Bim^{-/-}$ cells showed extended survival, whereas $Foxo3a^{-/-}$ and $Bim^{\Delta Foxo/\Delta Foxo}$ B cells died at similar rates as the wt B cells (FIG 5A, lower panel).

During shutdown of immune responses, a substantial fraction of activated T cells are killed to minimise the risk of immunopathology and to make space for subsequent immune responses against different pathogens[19]. Since Bim triggered by a reduction in

cytokine levels is essential for this death of activated T cells[10], Foxo transcription factors have been implicated as the critical inducers of *Bim* in this process[14]. We examined the importance of Foxo-mediated transcriptional induction of *Bim* in cytokine deprivation-induced killing of activated T cells. In the presence of IL-2, mitogen stimulated T cell blasts of all genotypes survived comparably. $Bim^{-/-}$ T cell blasts were almost completely protected from cytokine deprivation and those from the $Foxo3a^{-/-}$ mice showed modest (albeit significant) protection (FIG 5B). In contrast, the $Bim^{\Delta Foxo/\Delta Foxo}$ T cell blasts died at the same rate as wt cells (Figure 5B). Upon IL-2 withdrawal, *Bim* mRNA and Bim protein levels increased to similar extents in $Bim^{\Delta Foxo/\Delta Foxo}$, $Foxo3a^{-/-}$ and wt T cell blasts (FIG 5C).

In order to examine whether direct transcriptional induction of Bim by Foxo3a or related Foxo family members is critical for growth factor deprivation induced apoptosis of myeloid cells, we generated IL-3+SCF-dependent mast cells from the bone marrow of $Bim^{\Delta Foxo/\Delta Foxo}$, $Bim^{-/-}$, $Foxo3a^{-/-}$ and wt mice. As reported[20], mast cells from $Bim^{-/-}$ and $Foxo3a^{-/-}$ mice survived cytokine deprivation significantly better than their wt counterparts, but the $Bim^{\Delta Foxo/\Delta Foxo}$ mast cells died at a normal rate (FIG 5D). Interestingly, cytokine deprivation caused comparable levels of Bim protein upregulation in wt, $Foxo3a^{-/-}$ and $Bim^{\Delta Foxo/\Delta Foxo}$ mast cells (FIG 5E).

Collectively, these results show that direct induction of *Bim* mRNA expression by Foxo transcription factors is not essential for cytokine deprivation-induced apoptosis in diverse hematopoietic cell types.

Myc-driven lymphoma development is not affected by the $Bim^{\Delta Foxo}$ mutation

Both, the pro-apoptotic BH3-only protein Bim and Foxo transcription factors have been shown to act as tumor suppressors[17, 21]. Loss of both or even one allele of *Bim* substantially accelerates pre-B/B lymphoma development in *Eμ-myc* transgenic mice[22]. To test whether Foxo-mediated transcriptional induction of *Bim* is critical for its tumour suppressive function, we crossed the $Bim^{\Delta Foxo/\Delta Foxo}$ mice with the *Eμ-myc* transgenic mice[23] and monitored tumour development. There was no significant difference in the rate of lymphoma development or tumour immunophenotype (~60% $B220^{+}sIg^{-}$ pre-B and ~40% $B220^{+}sIg^{+}$ B) between *Eμ-myc* (control), $E\mu\text{-}myc;Bim^{\Delta Foxo/wt}$ and $E\mu\text{-}myc;Bim^{\Delta Foxo/\Delta Foxo}$ mice (supplementary FIG 3A & B). We therefore conclude that direct transcriptional induction of *Bim* by Foxo transcription factors is not critical for Bim's tumour suppressive function.

Discussion

The pro-apoptotic BH3 only Bcl-2 family member Bim is critical for several developmentally programmed pathways to cell death in the hematopoietic system [8][9][6]. Studies *in vitro* have shown that Bim is essential for the death of diverse cell types triggered by a broad range of apoptotic stimuli[6].

Despite the importance of Bim in so many processes, no transcriptional and/or post-transcriptional regulator has been shown to be critical in physiological settings *in vivo*. This is particularly the case for cytokine deprivation-induced apoptosis, a process that is critical for at least some of the functions of Bim within the whole animal[24]. Experiments using immortalised cytokine-dependent cell lines and enforced expression of dominant negative or constitutively active Foxo3a mutants have indicated that this

transcription factor binds within the two *Bim* promoters and is essential for the activation of Bim in cytokine deprivation-induced apoptosis[12-14]. It appears likely that the dominant negative mutant of Foxo3a can also inhibit transcriptional induction of target genes by the other Foxo family members, Foxo1, Foxo4 and Foxo6[17]. Therefore several of these transcription factors may be able to drive Bim induction in response to growth factor withdrawal.

To determine the impact of loss of transcriptional induction of *Bim* by all Foxo family members, we generated mice in which all four potential Foxo binding sites in the two *Bim* promoters had been mutated. In contrast to Bim-deficient cells[6], T as well as B lymphoid cells and mast cells from these animals were normally sensitive to cytokine deprivation. How can these results be reconciled with previous studies demonstrating a role for Foxo transcription factors in Bim activation in response to cytokine deprivation[12-14]? It remains theoretically possible that Foxo transcription factors can directly activate *Bim* expression by binding to sites other than the four that we have mutated. However, our sequence analysis and overexpression of Foxo3a in T cell blasts from $Bim^{\Delta Foxo/\Delta Foxo}$ mice (supplementary FIG 2C) did not reveal any additional candidate Foxo binding sites in the *Bim* promoter region. It also remains possible that Foxo proteins regulate Bim expression indirectly, for example by inducing another transcription factor that does bind to the *Bim* promoter or by controlling the expression of microRNAs that regulate Bim expression levels. In this regard it is noteworthy that the mir17-92 cluster has been shown to regulate apoptosis by repressing Bim expression[25]. It will therefore be interesting to examine whether the mir17-92 cluster can be regulated by Foxo transcription factors. Alternatively, cytokine deprivation may activate Bim by a

mechanism that is entirely independent of the Foxo transcription factors. This question might be resolved by generating cells that lack all Foxo family members, although such cells may be non-viable due to the loss of essential functions of Foxo transcription factors that are not related to transcriptional induction of *Bim*. It must also be noted that our studies were conducted exclusively with hematopoietic cells. *Bim* expression was reported to be regulated by Foxo transcription factors in neurons[26]; hence it will be interesting to use our $Bim^{\Delta Foxo/\Delta Foxo}$ mice to examine the physiological relevance of this finding. However, the mechanisms that control Bim expression in response to growth factor withdrawal in hematopoietic cells remain to be defined.

It is interesting that loss of Foxo3a could significantly delay spontaneous and dexamethasone induced thymocyte apoptosis and cytokine deprivation-induced apoptosis of T cell blasts and mast cells, although it did not reduce Bim expression. This indicates that Foxo3a must regulate a critical pro-apoptotic gene other than *Bim* under these stress conditions. Notably, Foxo3a was shown to up-regulate *Puma* expression in response to cytokine deprivation[27] and to cooperate with Bim in spontaneous and dexamethasone induced thymocyte killing, and IL-2 or IL-3+SCF withdrawal induced killing of T cell blasts or mast cells, respectively[7, 28]. This indicates that Foxo3a may play a critical role in these pathways to apoptosis by transcriptional induction of *Puma* (rather than *Bim*).

Finally, we showed that although Bim suppresses Myc-induced lymphomagenesis[22], mutation of all Foxo binding sites in the two *Bim* promoters did not alter the incidence or rate of pre-B/B lymphoma development in *Eμ-myc* transgenic mice. It remains possible that Foxo proteins regulate the expression and thereby the tumour suppressive action of

Bim indirectly (see above for possible processes). Alternatively, the activation of Bim during tumour suppression is mediated by a yet to be identified process that is independent of Foxo transcription factors.

Methods

see supplementary material section

Acknowledgements

We thank Dr P Coffer (UMC, Utrecht, Netherlands) for providing the Foxo3a(A3) plasmid, Dr H Puthalakath (LaTrobe University, Bundoora, VIC, Australia) for help with the EMSAs, Dr LA O'Reilly for reagents, Drs N Motoyama, S Cory and C Vandenberg for *Foxo3a*$^{-/-}$ mice, A Delbridge and Dr G Kelly for help with the lymphoma studies, K Hughes and J Coughlin for mouse husbandry, B Helbert and C Young for genotyping of mice and the WEHI flow cytometry facility for cell sorting. This work was supported by a postdoctoral fellowships from the German Research Council (He 5740/1-1) and the Leukaemia Foundation Australia (MJH), a MIRS scholarship from the University of Melbourne (LR), and Research Fellowships 1020363 (AS) and 1042629 (PB) from the National Health and Medical Research Council of Australia (NHMRC). PB and AS and their laboratories are supported by Program Grant 1016701 from the NHMRC and two Specialized Center of Research Grants from the Leukemia & Lymphoma Society (7001-13 and 7417-07). This work was made possible through Victorian State Government Operational Infrastructure Support and the Australian Government NHMRC Independent Research Institute Infrastructure Support Scheme.

Author contributions

MJH planned and conducted most experiments, interpreted results and wrote the manuscript. LR and MJL performed some experiments. LT performed gene-targeting in ES cells. TK, RG and SG performed the EMSA experiments. PB, TK and AS conceived study, planned experiments, interpreted data and wrote the manuscript.

Conflict of interest

All authors declare that they have no conflicts of interest in regards to this manuscript.

References

1. Strasser A, O'Connor L, Dixit VM (2000) Apoptosis signaling. *Ann Rev Biochem* **69:** 217-245

2. Youle RJ, Strasser A (2008) The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol* **9:** 47-59

3. Puthalakath H, Strasser A (2002) Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins. *Cell Death Differ* **9:** 505-512

4. Merino D *et al* (2009) The role of BH3-only protein Bim extends beyond inhibiting Bcl-2-like prosurvival proteins. *J Cell Biol* **186:** 355-362

5. O'Connor L, Strasser A, O'Reilly LA, Hausmann G, Adams JM, Cory S, Huang DCS (1998) Bim: a novel member of the Bcl-2 family that promotes apoptosis. *EMBO J* **17:** 384-395

6. Bouillet P, Metcalf D, Huang DCS, Tarlinton DM, Kay TWH, Köntgen F, Adams JM, Strasser A (1999) Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. *Science* **286:** 1735-1738

7. Erlacher M, Laabi V, Manzl C, Bock G, Tzankov A, Haecker G, Strasser A, Villunger A (2006) Puma cooperates with Bim, the rate-limiting BH3-only protein in cell death during lymphocyte development, in apoptosis induction. *Journal of Experimental Medicine* **203:** 2939-2951

8. Bouillet P *et al* (2002) BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes. *Nature* **415:** 922-926

9. Enders A, Bouillet P, Puthalakath H, Xu Y, Tarlinton DM, Strasser A (2003) Loss of the pro-apoptotic BH3-only Bcl-2 family member Bim inhibits BCR stimulation-induced apoptosis and deletion of autoreative B cells. *J Exp Med* **198:** 1119-1126

10. Pellegrini M, Belz G, Bouillet P, Strasser A (2003) Shut down of an acute T cell immune response to viral infection is mediated by the pro-apoptotic Bcl-2 homology 3-only protein Bim. *Proc Natl Acad Sci U S A* **100:** 14175-14180

11. Fischer SF, Bouillet P, O'Donnell K, Light A, Tarlinton DM, Strasser A (2007) Pro-apoptotic BH3-only protein Bim is essential for developmentally programmed death of germinal center-derived memory B cells and antibody forming cells. *Blood* **110:** 3978-3984

12. Dijkers PF, Birkenkamp KU, Lam EW, Thomas NS, Lammers JW, Koenderman L, Coffer PJ (2002) FKHR-L1 can act as a critical effector of cell death induced by

cytokine withdrawal: protein kinase B-enhanced cell survival through maintenance of mitochondrial integrity. *Journal of Cell Biology* **156:** 531-542

13. Dijkers PF, Medema RH, Lammers JJ, Koenderman L, Coffer PJ (2000) Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. *Current Biology* **10:** 1201-1204.

14. Stahl M, Dijkers PF, Kops GJ, Lens SM, Coffer PJ, Burgering BM, Medema RH (2002) The forkhead transcription factor FoxO regulates transcription of $p27^{Kip1}$ and Bim in response to IL-2. *Journal of Immunology* **168:** 5024-5031

15. Gaviraghi M, Caricasole A, Costanzo C, Diamanti D, Dandrea M, Donadelli M, Scarpa A, Palmieri M (2008) Identification of a candidate alternative promoter region of the human Bcl2L11 (Bim) gene. *BMC Mol Biol* **9:** 56

16. Pellegrini M, Bouillet P, Robati M, Belz GT, Davey GM, Strasser A (2004) Loss of Bim Increases T Cell Production and Function in Interleukin 7 Receptor-deficient Mice. *J Exp Med* **200:** 1189-1195

17. Fu Z, Tindall DJ (2008) FOXOs, cancer and regulation of apoptosis. *Oncogene* **27:** 2312-2319

18. Maraskovsky E, O'Reilly LA, Teepe M, Corcoran LM, Peschon JJ, Strasser A (1997) Bcl-2 can rescue T lymphocyte development in interleukin-7 receptor-deficient mice but not in mutant *rag-1* $^{-/-}$ mice. *Cell* **89:** 1011-1019

19. Strasser A, Pellegrini M (2004) T-lymphocyte death during shutdown of an immune response. *Trends Immunol* **25:** 610-615

20. Moller C, Alfredsson J, Engstrom M, Wootz H, Xiang Z, Lennartsson J, Jonsson JI, Nilsson G (2005) Stem cell factor promotes mast cell survival via inactivation of

FOXO3a-mediated transcriptional induction and MEK-regulated phosphorylation of the proapoptotic protein Bim. *Blood* **106:** 1330-1336

21. Cragg MS, Harris C, Strasser A, Scott CL (2009) Unleashing the power of inhibitors of oncogenic kinases through BH3 mimetics. *Nat Rev Cancer* **9:** 321-326

22. Egle A, Harris AW, Bouillet P, Cory S (2004) Bim is a suppressor of Myc-induced mouse B cell leukemia. *Proc Natl Acad Sci U S A* **101:** 6164-6169

23. Adams JM, Harris AW, Pinkert CA, Corcoran LM, Alexander WS, Cory S, Palmiter RD, Brinster RL (1985) The c-*myc* oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. *Nature* **318:** 533-538

24. Strasser A (2005) The role of BH3-only proteins in the immune system. *Nat Rev Immunol* **5:** 189-200

25. Ventura A *et al* (2008) Targeted deletion reveals essential and overlapping functions of the mIR-17~92 family of miRNA clusters. *Cell* **132:** 875-886

26. Gilley J, Coffer PJ, Ham J (2003) FOXO transcription factors directly activate *bim* gene expression and promote apoptosis in sympathetic neurons. *Journal of Cell Biology* **162:** 613-622

27. You H, Pellegrini M, Tsuchihara K, Yamamoto K, Hacker G, Erlacher M, Villunger A, Mak TW (2006) FOXO3a-dependent regulation of Puma in response to cytokine/growth factor withdrawal. *J Exp Med* **203:** 1657-1663

28. Ekoff M, Kaufmann T, Engstrom M, Motoyama N, Villunger A, Jonsson JI, Strasser A, Nilsson G (2007) The BH3-only protein Puma plays an essential role in cytokine deprivation-induced apoptosis of mast cells. *Blood* **110:** 3209-3217

Figure Legends

Figure 1 Identification of candidate Foxo binding sites in the *Bim* promoters. (**A**) Map showing four Foxo binding sites within the two *Bim* promoters. (**B**) Electrophoretic mobility shift assay (EMSA) to test for binding of Foxo3a to the four Foxo binding sites in the *Bim* promoters. ^{32}P-labelled oligos corresponding to sites 1, 2, 3 or 4 were incubated with nuclear extracts from 293T cells transiently transfected with a control plasmid or a plasmid encoding tamoxifen inducible active HA-tagged Foxo3a (HA-Foxo3A(A3)ER) that had been treated for 24 h with tamoxifen (to activate Foxo3a). (**C**) Control and HA-Foxo3a(A3)ER nuclear extracts were left untreated or incubated with either HA or Foxo3A specific antibodies to verify the specificity of Foxo3A binding to site 4 in the *Bim* promoters. Arrows indicate specific bands in (**B**) and (**C**).

Figure 2 *Bim*$^{\Delta Foxo/\Delta Foxo}$ mice have normal T lymphoid cell subset composition in the thymus. (**A**) Cell numbers of $CD4^-8^-$ progenitors, $CD4^+8^+$ immature, $CD4^+8^-$ and $CD4^-8^+$ mature thymocytes were determined by flow cytometry in wt, *Bim* $^{\Delta Foxo/\Delta Foxo}$, *Foxo3a*$^{-/-}$ and *Bim*$^{-/-}$ mice (n indicates the numbers of mice analysed for each genotype). The data represent means +/-SD; * $p<0.05$. (**B**) Western blot analysis to determine the levels of Bim protein expression in extracts from thymocytes of wt, *Bim* $^{\Delta Foxo/\Delta Foxo}$, *Foxo3a*$^{-/-}$ and *Bim*$^{-/-}$ mice. Probing for β-actin served as a loading control. (**C**) qRT-PCR analysis to determine the levels of *Bim* mRNA expression in extracts from thymocytes of wt, *Bim*$^{\Delta Foxo/\Delta Foxo}$ and *Foxo3a*$^{-/-}$ mice. The expression of *Bim* mRNA was normalised to HMBS mRNA. The data represent means +/- SD; ** $p<0.01$.

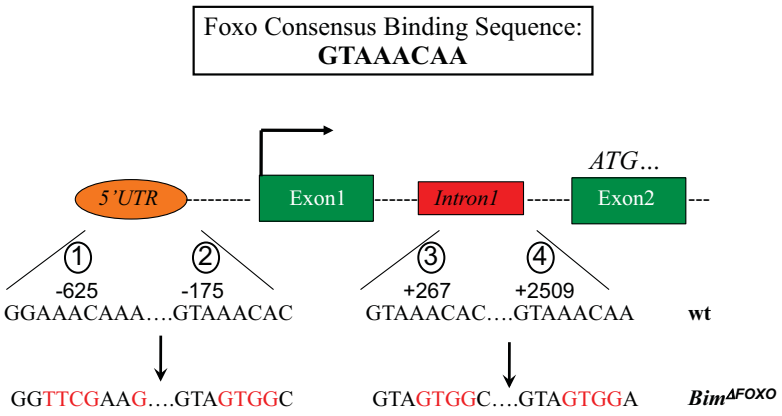
Figure 3 *Bim*$^{\Delta Foxo/\Delta Foxo}$ mice have normal hematopoietic cell subset composition in the spleen. (**A**) The numbers of B cells, $CD4^+8^-$ as well as $CD4^-8^+$ mature T cells, macrophages ($Mac\text{-}1^+Gr\text{-}1^-$) and granulocytes ($Mac\text{-}1^+Gr\text{-}1^+$) in the spleens of wt, *Bim* $^{\Delta Foxo/\Delta Foxo}$, *Foxo3a*$^{-/-}$ and *Bim*$^{-/-}$ mice were determined by flow cytometry (n indicates the numbers of mice for each genotype analysed). The data represent means +/-SD; * $p<0.05$, *** $p<0.0005$, **** $p<0.0001$. (**B**) Western blot analysis to determine the levels of Bim protein expression in extracts from spleen cells of wt, *Bim* $^{\Delta Foxo/\Delta Foxo}$, *Foxo3a*$^{-/-}$ and *Bim*$^{-/-}$ mice. Probing for HSP70 served as a loading control. (**C**) qRT-PCR analysis to determine the levels of *Bim* mRNA expression in extracts from spleen cells of wt, *Bim*$^{\Delta Foxo/\Delta Foxo}$ and *Foxo3a*$^{-/-}$ mice. The expression of *Bim* mRNA was normalised to HMBS mRNA.

Figure 4 Thymocytes from *Bim*$^{\Delta Foxo/\Delta Foxo}$ mice are normally sensitive to a broad range of apoptotic stimuli that kill these cells in a Bim-dependent manner. Survival of thymocytes from wt, *Bim* $^{\Delta Foxo/\Delta Foxo}$, *Foxo3a*$^{-/-}$ and *Bim*$^{-/-}$ mice in culture, either in simple medium (no added growth factors, to simulate growth factor withdrawal) or after treatment with dexamethasone, ionomycin or phorbol ester (PMA), was determined at the indicated time points by flow cytometry (n indicates numbers of mice from each genotype). The data represent means +/-SD; * $p<0.05$, ** $p<0.01$, *** $p<0.0005$, **** $p<0.0001$.

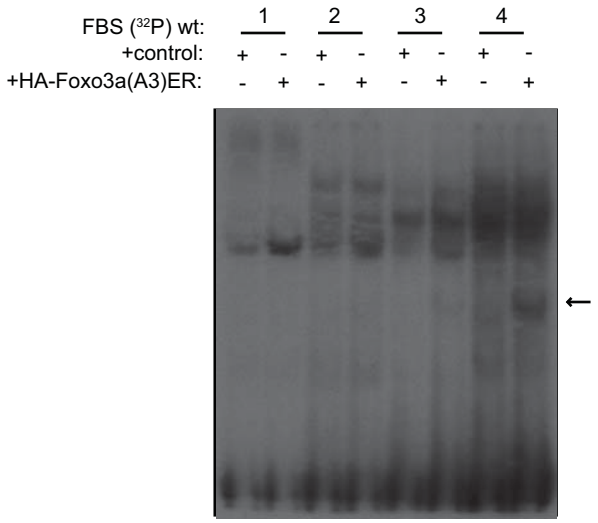
Figure 5 Diverse lymphoid as well as mast cells from *Bim*$^{\Delta Foxo/\Delta Foxo}$ mice are normally sensitive to cytokine deprivation. (**A**) Survival of FACS-sorted $CD4^+8^-$ or $CD4^-8^+$ mature T cells and transitional or follicular B cells from the spleens of wt, *Bim* $^{\Delta Foxo/\Delta Foxo}$, *Foxo3a*$^{-/-}$ and *Bim*$^{-/-}$ mice in culture in simple medium (no added growth factors) was

determined at the indicated time points by flow cytometry (n indicates numbers of mice from each genotype). The data represent means +/-SD; * $p<0.05$, ** $p<0.01$, *** $p<0.0005$, **** $p<0.0001$. (**B**) Survival of mitogen-stimulated T cell blasts from wt, *Bim* $^{\Delta Foxo/\Delta Foxo}$, *Foxo3a*$^{-/-}$ and *Bim*$^{-/-}$ mice in culture in simple medium (after removal of IL-2) was determined at the indicated time points by flow cytometry (n indicates numbers of mice from each genotype). The data represent means +/-SD; * $p<0.05$, ** $p<0.01$, *** $p<0.0005$, **** $p<0.0001$. (**C**) qRT-PCR and Western blot analyses to determine the levels of *Bim* mRNA and Bim protein expression, respectively, in the T cell blasts described in **B**, in the presence of IL-2 or 8 h after removal of IL-2 (in the presence of the pan-caspase inhibitor QVD-OPH (20 μM) to prevent RNA and protein degradation). For qRT-PCR analysis *Bim* mRNA levels were normalised to HMBS. Probing for HSP70 served as a loading control. (**D**) Survival of IL-3+SCF-dependent mast cells from wt, *Bim* $^{\Delta Foxo/\Delta Foxo}$, *Foxo3a*$^{-/-}$ and *Bim*$^{-/-}$ mice in simple medium (after removal of IL-3+SCF) was determined at the indicated time points by flow cytometry (n indicates numbers of mice from each genotype). The data represent means +/-SD; ** $p<0.01$, *** $p<0.0005$, **** $p<0.0001$. (**E**) Western blot analysis to determine the levels of Bim protein expression in the mast cells described in **D**, in the presence of IL-3+SCF or 8 h after removal of these growth factors (in the presence of the pan-caspase inhibitor QVD-OPH (20 μM) to prevent RNA and protein degradation). Probing for HSP70 served as a loading control.

A


Foxo Consensus Binding Sequence:
GTAAACAA
ATG…
5'UTR
Exon1
Intron1
Exon2
①
②
③
④
-625
-175
+267
+2509
GGAAACAAA….GTAAACAC
GTAAACAC….GTAAACAA
wt
GGTTCGAAG….GTAGTGGC
GTAGTGGC….GTAGTGGA
Bim^ΔFOXO

B


FBS (32P) wt:
1
2
3
4
+control:
+HA-Foxo3a(A3)ER:

C

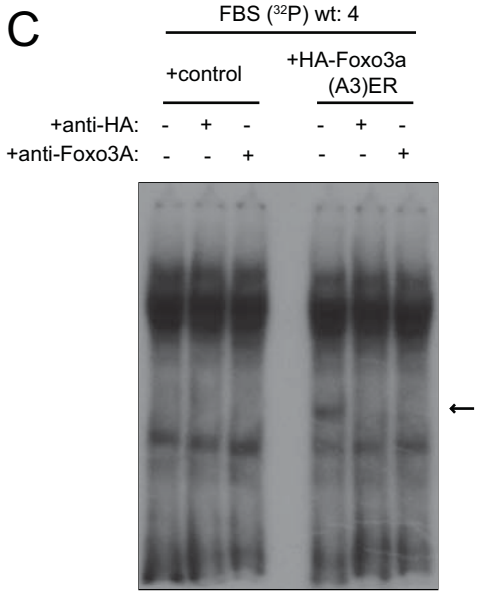

FBS (32P) wt: 4
+control
+HA-Foxo3a (A3)ER
+anti-HA:
+anti-Foxo3A:

Figure 1

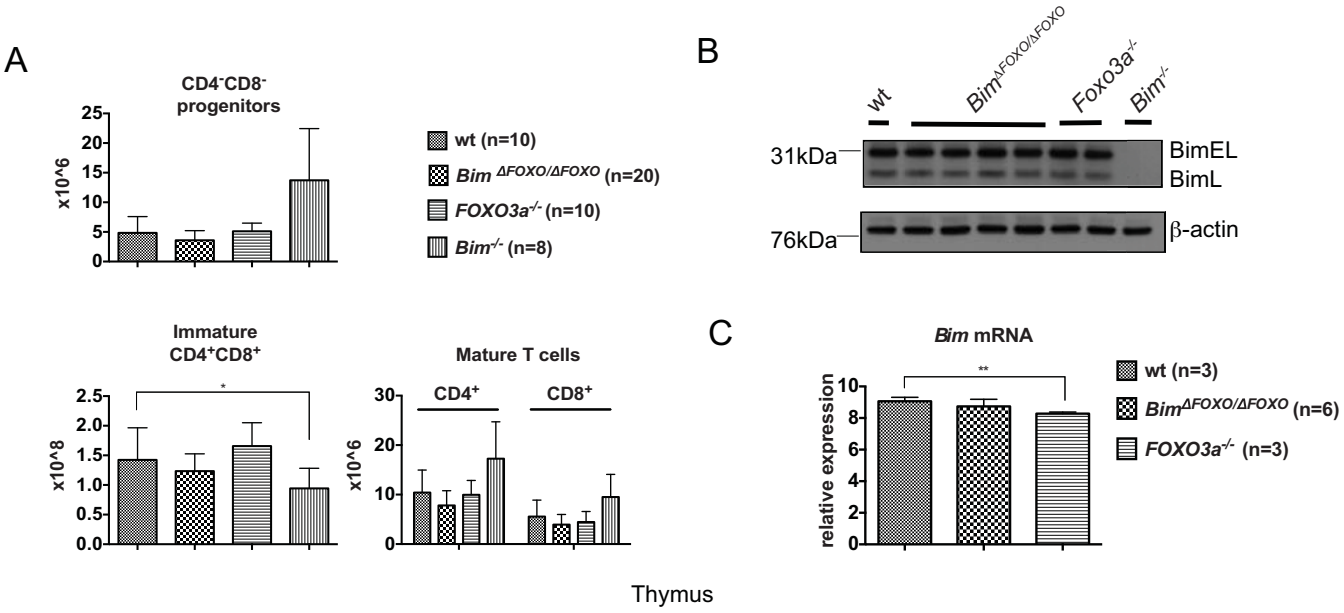

A
CD4-CD8- progenitors
x10^6
25
20
15
10
5
0
wt (n=10)
Bim ΔFOXO/ΔFOXO (n=20)
FOXO3a-/- (n=10)
Bim-/- (n=8)
Immature CD4+CD8+
x10^8
2.5
2.0
1.5
1.0
0.5
0.0
*
Mature T cells
CD4+
CD8+
x10^6
30
20
10
0
B
wt
BimΔFOXO/ΔFOXO
Foxo3a-/-
Bim-/-
31kDa
BimEL
BimL
76kDa
β-actin
C
Bim mRNA
relative expression
10
8
6
4
2
0
**
wt (n=3)
BimΔFOXO/ΔFOXO (n=6)
FOXO3a-/- (n=3)
Thymus

Figure 2

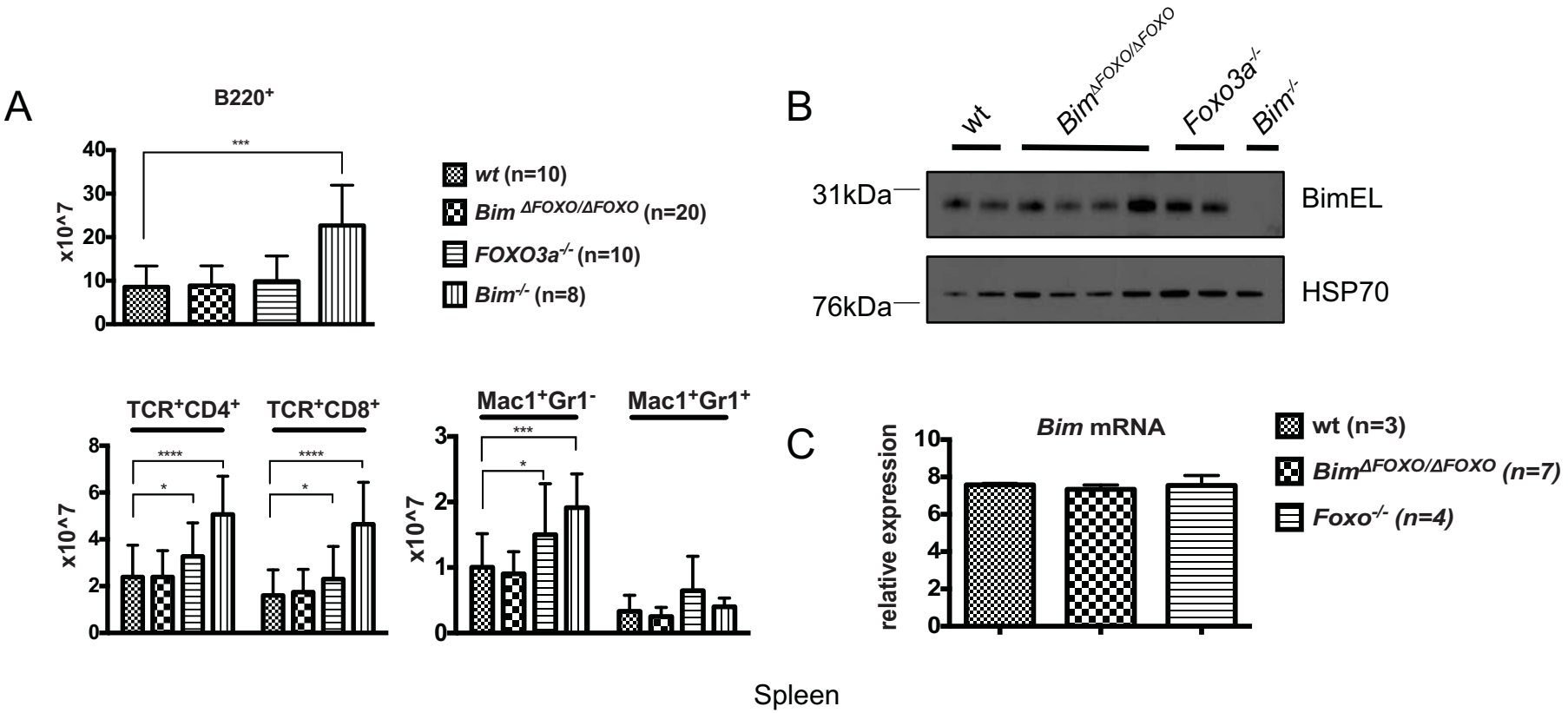

A
B220+
x10^7
wt (n=10)
Bim ΔFOXO/ΔFOXO (n=20)
FOXO3a-/- (n=10)
Bim-/- (n=8)
TCR+CD4+
TCR+CD8+
Mac1+Gr1-
Mac1+Gr1+
B
wt
BimΔFOXO/ΔFOXO
Foxo3a-/-
Bim-/-
31kDa
BimEL
76kDa
HSP70
C
Bim mRNA
relative expression
wt (n=3)
BimΔFOXO/ΔFOXO (n=7)
Foxo-/- (n=4)
Spleen

Figure 3

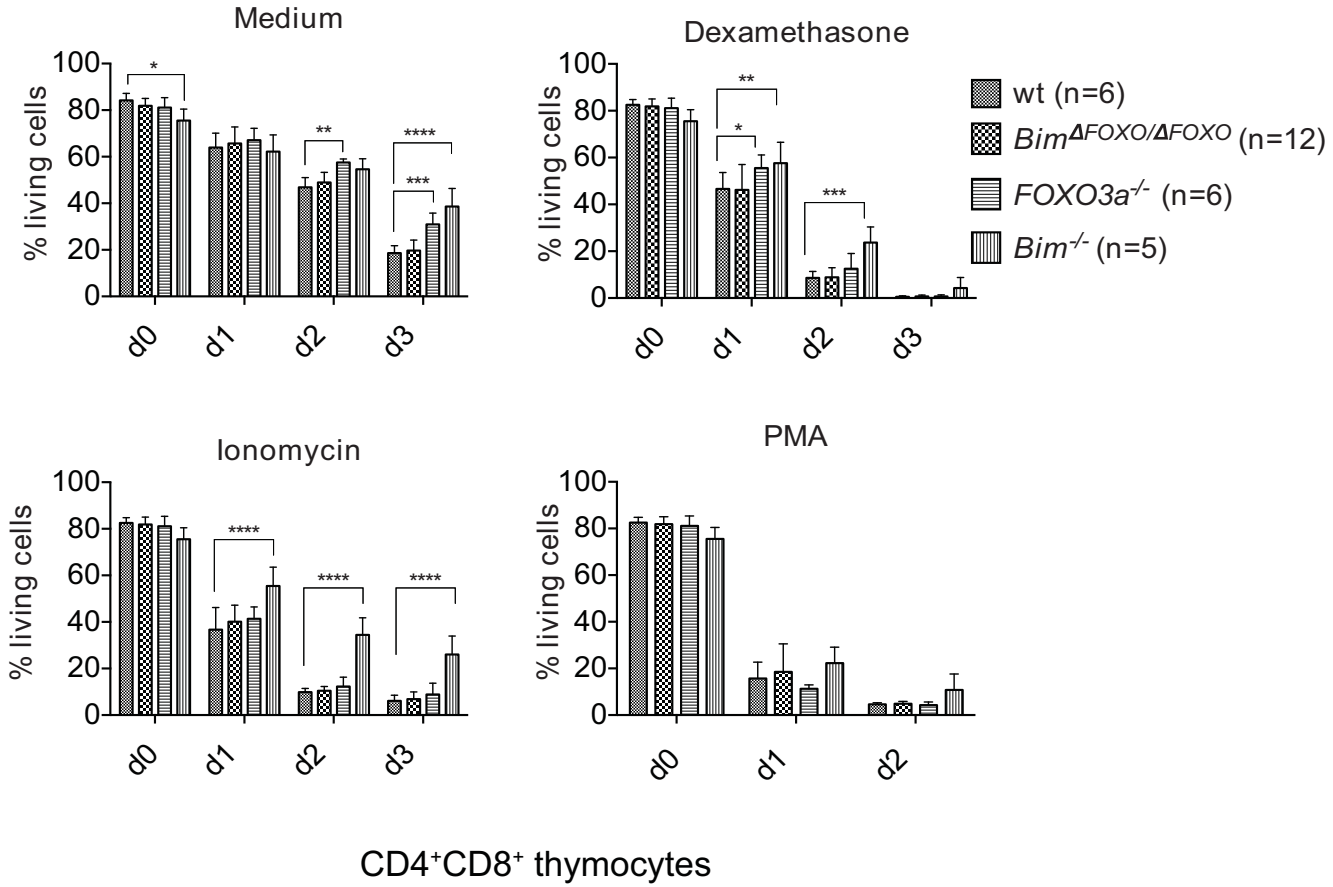

Medium
Dexamethasone
Ionomycin
PMA
% living cells
100
80
60
40
20
0
d0
d1
d2
d3
*
**
***
****
wt (n=6)
Bim^ΔFOXO/ΔFOXO (n=12)
FOXO3a^-/- (n=6)
Bim^-/- (n=5)
CD4+CD8+ thymocytes

Figure 4

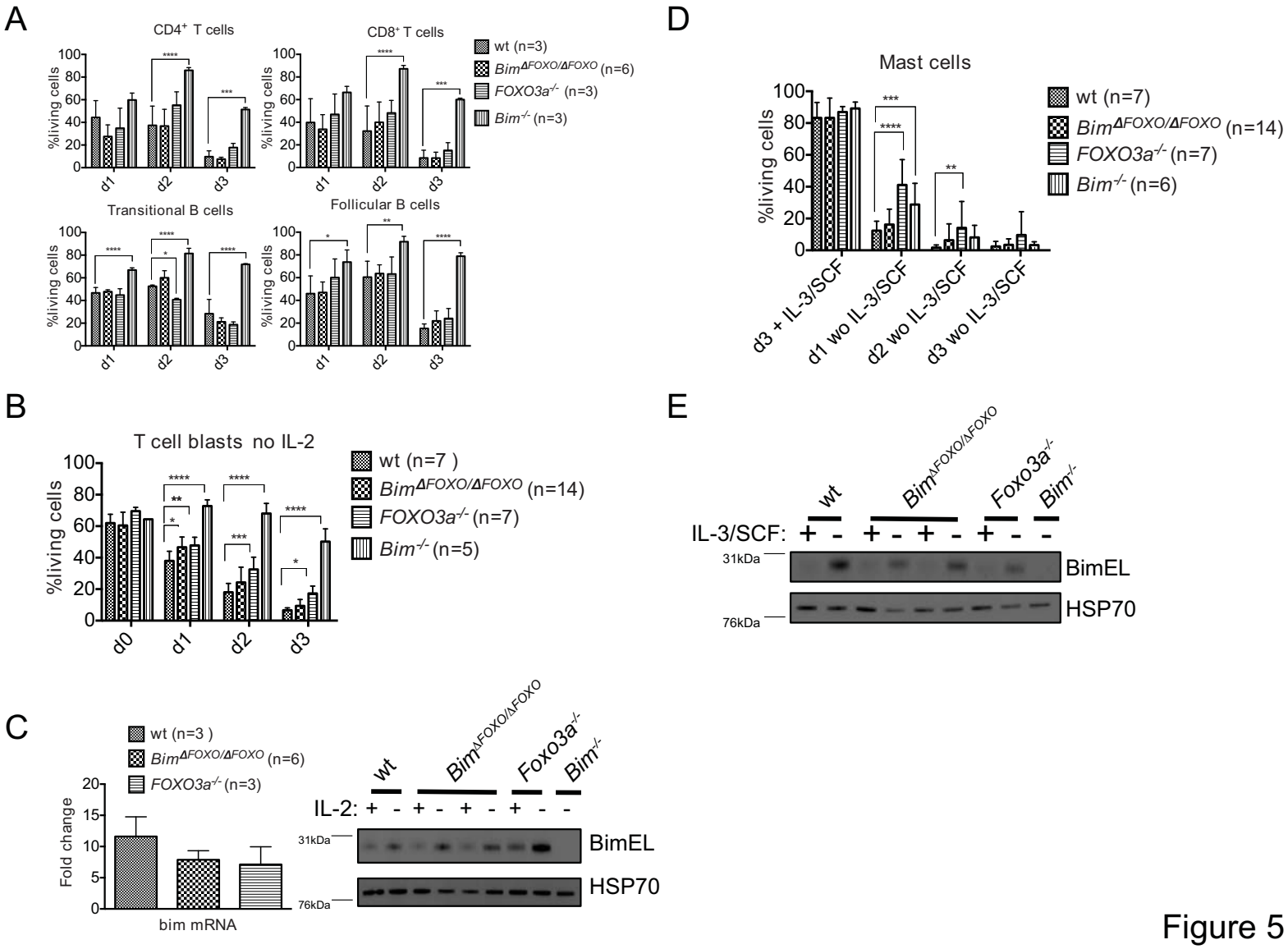

A
CD4+ T cells
CD8+ T cells
Transitional B cells
Follicular B cells
%living cells
d1
d2
d3
wt (n=3)
Bim^ΔFOXO/ΔFOXO (n=6)
FOXO3a-/- (n=3)
Bim-/- (n=3)
B
T cell blasts no IL-2
d0
wt (n=7)
Bim^ΔFOXO/ΔFOXO (n=14)
FOXO3a-/- (n=7)
Bim-/- (n=5)
C
wt (n=3)
Bim^ΔFOXO/ΔFOXO (n=6)
FOXO3a-/- (n=3)
Fold change
bim mRNA
wt
Bim^ΔFOXO/ΔFOXO
Foxo3a-/-
Bim-/-
IL-2:
31kDa
76kDa
BimEL
HSP70
D
Mast cells
wt (n=7)
Bim^ΔFOXO/ΔFOXO (n=14)
FOXO3a-/- (n=7)
Bim-/- (n=6)
d3 + IL-3/SCF
d1 wo IL-3/SCF
d2 wo IL-3/SCF
d3 wo IL-3/SCF
E
wt
Bim^ΔFOXO/ΔFOXO
Foxo3a-/-
Bim-/-
IL-3/SCF:
31kDa
76kDa
BimEL
HSP70

Figure 5